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Partitioning and separation of α-lactalbumin and β-lactoglobulin in polyethylene glycol/ammonium sulphate aqueous two-phase systems

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Abstract

The partition behaviour of α-lactalbumin (αla) and β-lactoglobulin (βlg) on PEG/$(NH_4)_2SO_4$ system was studied. For purified proteins, a partition coefficient of 12.8 for αla and 0.34 for βlg, with mass recovery yields of 96.7% for αla in the upper phase and 83.8% for βlg in the lower phase was obtained, in 18% (w/w) PEG 900/14% (w/w) $(NH_4)_2SO_4$ system, at pH 7. PEG/$(NH_4)_2SO_4$ system was an economical alternative for the recovery and separation of the two proteins in cheese whey, allowing a 50% reduction in costs. An efficient and inexpensive separation of both proteins in cheese whey could be achieved, by using 16% (w/w) PEG 900/15% (w/w) $(NH_4)_2SO_4$, at pH 7.5.

Introduction

The purification of biological materials is usually done by precipitation with salts or organic solvents and various chromatographic techniques. However, these techniques carry enormous difficulties when considering their use in large-scale applications (Alves *et al.* 2000). Liquid-liquid extraction in aqueous two-phase systems (ATPS) has proved to be a promising separation strategy for many biological products (Andrews *et al.* 2000).

ATPS, such as polyethylene glycol PEG/citrate, PEG/phosphate and PEG/sulphate, are best for continuous large-scale purification of biological origin materials and allow the use of traditional liquid-liquid extraction equipment (Coimbra *et al.* 1995).

Protein purification using ATPS is influenced by several parameters, such as the pH of the system, the type and concentration of salts in the system, the polymer's molecular mass and concentration, and properties of the protein (e.g., structure, hydrophobicity, molecular mass) (Albertsson 1985). Applications of ATPS to protein separation include the separation of proteins from cheese whey (Coimbra *et al.* 1994, 1995, Chen 1992). Cheese whey has a high protein content (12% w/w; dry basis), being α-lactalbumin-αla-(20% w/w; dry basis) and β-lactoglobulin-βlg-(50% w/w; dry basis) the major protein constituents (Alves *et al.* 2000, Chen 1992, Pearce 1992). Since cheese whey protein has a high nutritional value and excellent functional properties (Pearce 1992), it has recently been considered to be used in the food industry.

In the present work, systems composed of PEG and ammonium sulphate were studied, for the separation of αla and βlg (from whey protein concentrate). The main factors affecting the proteins partition behaviour were first evaluated, aiming at determining the optimal separation conditions. Assays were then done with a whey protein concentrate. The obtained results were compared with work done by Chen (1992) and Alves *et al.* (2000), on the separation of the major proteins from cheese whey by aqueous two-phase systems (where PEG and potassium phosphate systems were used).

Materials and methods

Chemicals

PEG with average molecular masses between 600 and 900 Da were purchased from Sigma; PEG with average molecular mass of 1500 Da was purchased from Merck.

Proteins

αla and βlg were purchased from Sigma. Whey protein concentrate (WPC) with 80% (w/w; dry basis) protein was obtained from Quinta dos Ingleses, Lda.

Two-phase systems

The systems were prepared from stock solutions of polymers and salt in water. PEG and $(NH_4)_2SO_4$ stock solutions were prepared with a concentration of 50% (w/w) (in distilled water). The systems were prepared from stock solutions of PEG and $(NH_4)_2SO_4$.

For all tested systems (purified proteins and WPC), several PEG/$(NH_4)_2SO_4$ systems were tested for their ability to separate αla and βlg. The polymer composition of the systems was 14, 16 and 18% (w/w) and the $(NH_4)_2SO_4$ remained constant at 14% (w/w) in all assays.

For system preparation with purified proteins, polymer and salt stock solutions were weighed and mixed with distilled water, phosphate buffer at 10 mM (the tested systems pH were 6, 7 and 8) and a 0.5 mg protein (αla or βlg) ml^{-1}. To assay the effect of different salts in the partition, direct dissolution of the salts in the systems was provided. The total mass of the systems was adjusted to 1 g with distilled water.

For system preparation with WPC, polymer and salt stock solutions were weighed and mixed with distilled water, phosphate buffer at 10 mM (also at pH 6, 7 and 8) and 10% (w/w) WPC solution. The total mass of the systems was adjusted to 10.0 g with distilled water.

Phases were separated by centrifugation (5 min, 4000 *g*) (Chen 1992). Total mass and protein content were determined in both phases.

Protein assays

For the purified proteins protein concentration, in both phases of the system, was determined according to the Bradford method.

Previous work done on the separation of cheese whey proteins in ATPS, demonstrated the preferential partition of αla in the upper phase and βlg in the lower phase of the PEG/salts systems (Alves *et al.* 2000, Chen 1992).

To describe αla and βlg partition, two parameters were determined: the protein partition coefficient, defined as the ratio between the protein upper and lower phase concentrations ($K_{\alpha la}$ and $K_{\beta lg}$), the αla mass recovery yield for the upper phase ($Y_{\alpha la,U}$) and the βlg mass recovery yield for the lower phase ($Y_{\beta lg,L}$) (Chen 1992).

In the assays done with WPC, the proteins' partitioning behaviour (αla and βlg) was evaluated by ionic exchange chromatography. The phase samples (100 μl) were injected into a Mono Q HR5/5 column (Pharmacia). Assays were run at a flow rate of 1 ml min^{-1}, at room temperature, using 20 mM Tris/HCl buffer at pH 8, with an NaCl gradient (0–1 M) as eluent. Absorbancy at 280 nm was recorded, using a L-7455 Diode-Array detector (Merk), and analysed with D-7000 HPLC System Manager (Version 3.1) Software.

Results and discussion

PEG/$(NH_4)_2SO_4$ systems

PEG molecular mass effect on proteins partition

Figure 1 shows the effect of different molecular masses of PEG on the partitioning of αla and βlg. Partition coefficients of both proteins decreased with increasing PEG molecular mass. The proteins moved into the salt-rich lower phase, as the PEG molecular mass increased, possibly due to an excluded volume effect (Chen 1992).

The αla partition coefficient ($K_{\alpha la}$) (Figure 1) is greater than 1, indicating that αla is mainly in the upper phase of the systems. The βlg partition coefficient ($K_{\beta lg}$) ranges from 0.09 to 0.38, indicating that βlg is mainly in the lower phase of the systems. Partition results (Figure 1) shown that PEG 900 gives the highest separation of αla and βlg.

Chen (1992) suggested that the partition of a protein in PEG/salt ATPS depends on the hydrophobicity of the proteins. Proteins with more apolar amino acid residues will show higher affinity for the PEG phase (which is more hydrophobic than the lower salt phase). αla and βlg have similar hydrophobicities (4.68 and 5.03 kJ/(apolar residue), respectively) (Melander & Horváth 1977) and similar molecular masses

Table 1. Effect of pH on the partition of αla and βlg. Composition of the systems: 14% (w/w) $(NH_4)_2SO_4$ with PEG 900 at those concentrations.

pH	$K_{\alpha la}$			$K_{\beta lg}$		
	14% (w/w) PEG	16% (w/w) PEG	18% (w/w) PEG	14% (w/w) PEG	16% (w/w) PEG	18% (w/w) PEG
6	6	20.9	31.53	0.17	0.12	0.42
7	5.21	10.5	12.84	0.12	0.18	0.34
8	7.46	8.21	9.1	0.39	0.46	1.16

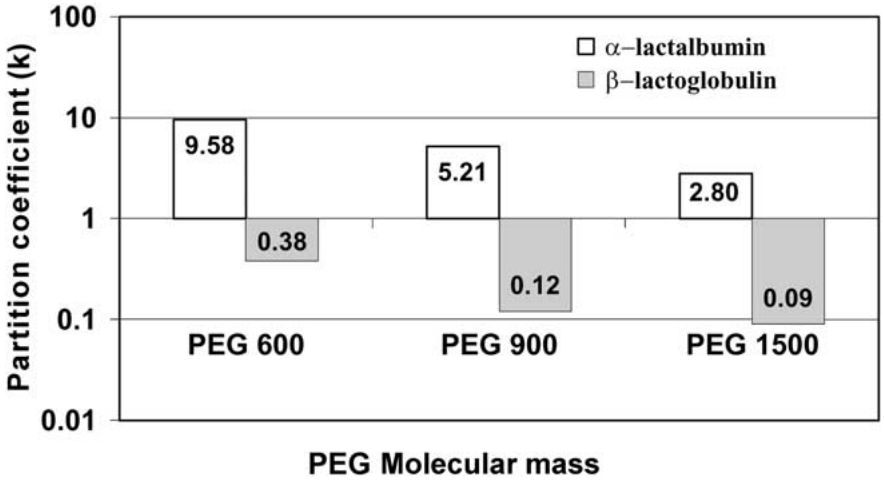

Fig. 1. Effect of different molecular masses of PEG on the partitioning of αla and βlg. Composition of the systems: 14% (w/w) $(NH_4)_2SO_4$/14% (w/w) PEG, at pH 7.

(αla = 14 kDa and βlg = 18 kDa) (Albertsson 1985). In addition, dipeptides containing tryptophan, partition strongly in favour of the PEG-rich upper phase in PEG/salt systems (Chen 1992). Since tryptophan represents 5.2 mol% of total amino acid residues in αla, in contrast to 2 mol% in βlg, this interaction may play a role in determining the partition behaviour observed (Chen 1992).

PEG concentration effect on proteins partition

The increase in K with the increase in PEG concentration may result from changes in the specific volume of the phases. As more PEG is added to the system, the specific volume of the upper phase remains approximately constant while the specific volume of the lower phase decreases rapidly (Ananthapadmanabhan & Goddard 1987). Hence the water molecules available for solute solvatation in the lower phase decrease and the proteins reach their solubility limit, being forced to partition to the upper phase (Figure 2). Results shown in Figure 2 suggest that 18% (w/w) of PEG 900 allowed the highest separation of the proteins.

pH effect on proteins partition

The effect of the system pH, on the partition of αla and βlg, for systems composed of 14% (w/w) $(NH_4)_2SO_4$ and 14, 16 and 18% (w/w) PEG 900, was assessed (Table 1). At a given pH, for both proteins, the partition coefficients increased with PEG concentration. However as the system pH was increased, K values decreased, this effect being more pronounced for αla. These results suggest that the pH is also determinant in the partition of these proteins. For αla, K values decreased significantly, as pH varied from 6 to 7. For βlg, major differences were detected between pH 7 and 8 (with the highest K values at pH 8).

In PEG/salt systems, PEG is overall positively charged, due to the polymer's repeating ether oxygen atoms. These are capable of ionic binding with the proteins' metallic elements (Chen 1992). Above the isoelectric point (αla = 5.2; βlg = 4.2–4.8; Chen 1992),

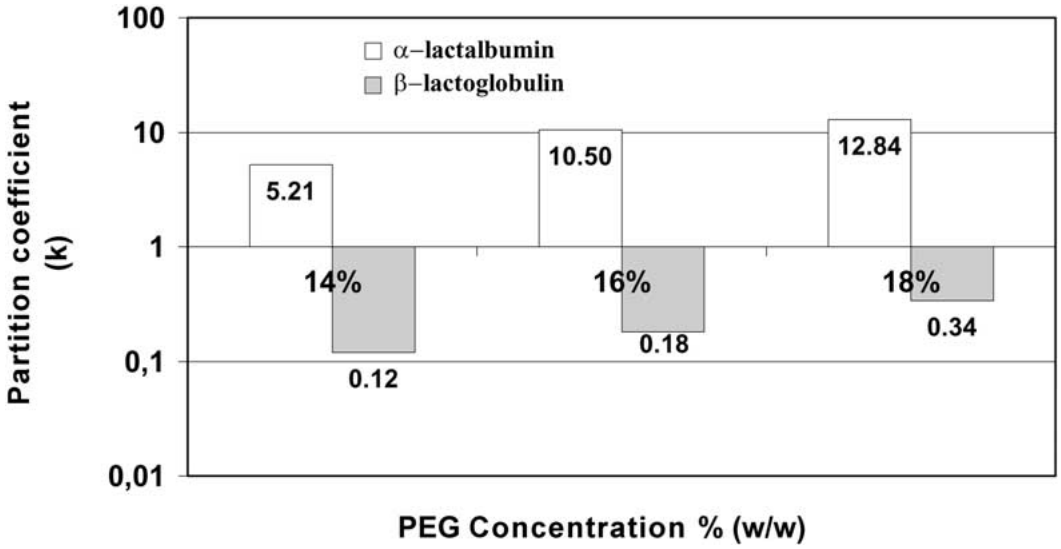

Fig. 2. Effect of PEG concentration on the partitioning of αla and βblg. Composition of the systems: 14% (w/w) $(NH_4)_2SO_4$/PEG900, at pH 7.

these proteins should be accepted more favourably by the PEG phase as the solution pH increases. However, the opposite was observed for αla (Table 1). Possibly, in the PEG/sulphate system here studied, as the pH increased, the protein gradually denaturised, thus precipitating (this was visually observed).

The effect of the addition of salts to PEG/$(NH_4)_2SO_4$ system on proteins partition

In a system composed of 16% (w/w) PEG900/14% (w/w) $(NH_4)_2SO_4$ at pH 7, the effect of the addition of different salts (NaCl, K_2SO_4 and KSCN) on αla and βlg partition, was determined.

In ATPS, salts can alter effectively the partition behaviour of proteins, due to a hydrophobicity effect (Cascone *et al.* 1991, Schmidt *et al.* 1994). As expected, the addition of salts to the PEG/$(NH_4)_2SO_4$ system, decreased the partition coefficients of both proteins (especially for KSCN) (data not shown), although this did not improve the proteins separation.

Results gathered from the assays done with pure proteins suggest that a system of 18% (w/w) PEG 900/14% (w/w) $(NH_4)_2SO_4$ at pH 7, allowed the highest recovery of the proteins. Mass recovery yields of 96.7% for αla in the upper phase and 83.8% for βlg in the lower phase, and partition coefficients of $K_{\alpha la} = 12.8$ and $K_{\beta lg} = 0.34$ were obtained.

Separation of αla and βlg in whey protein concentrate

The assays done with the pure proteins were repeated for whey protein concentrate, also aiming to determine the best operational conditions for the separation of αla and βlg, in a one-step liquid–liquid extraction. With WPC, the best separation conditions were 14% (w/w) PEG900/14% (w/w) $(NH_4)_2SO_4$ at pH 7, and 16% (w/w) PEG900/15% (w/w) $(NH_4)_2SO_4$ at pH 7.5, being different from the ones obtained with the pure proteins, although the tendencies observed for pure proteins were also observed.

With the above systems, purification of all βlg in the lower phase was achieved. This is due to βlg exclusive partition to the lower phase, no other protein being detected. These systems also allow the concentration of αla in the upper phase, although contamination with BSA occurred.

The partition coefficient values obtained with WPC (data not shown) were lower than the ones of the pure proteins. This difference was expected since whey has a complex composition (Pearce 1992, Palomares & Hernandez 1998).

To what concerns recovery and separation of the two major proteins in cheese whey, PEG/sulphate system may be an economical alternative to PEG/phosphate. In fact, results here gathered show that PEG/sulphate and PEG/phosphate systems (Alves *et al.* 2000, Coimbra *et al.* 1994, 1995, Chen 1992) have similar partition characteristics. In addition, sul-

phate has a lower price, allowing a 50% costs reduction.

Conclusions

PEG/$(NH_4)_2SO_4$ aqueous two-phase system proved to be an efficient and inexpensive system for αla and βlg separation from cheese whey, being shown that its possible to purify βlg in the lower phase and to concentrate αla in the upper phase of the systems.

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